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A novel sensitive immunoassay by nucleic acid barcode dot and its application in the detection of prostate-specific antigen

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Abstract

Background: The sensitivity and selectivity of traditional methods limits ultramicro detection of proteins. Bio-barcode amplification detection methods based on nanotechnology enables ultramicro detection of protein. However, bio-barcode amplification detection depends on the oligonucleotides being fixed on a glass chip. It also requires specialized equipment, which limits its application. We introduce a nano-nucleic acid barcode dot detection technology to determine ultramicro concentrations of protein. The method is simple, quick and accurate.

Methods: Magnetic probe (IgG-M) and dual-labeled gold nanoparticle bio-probe (IgG-Au-DNA) were prepared. Protein was captured using a sandwich assay technique and magnetic separation was used. The DNA barcode was released with dithiothreitol (DTT) and detected directly without the requirement for polymerase chain reaction (PCR). Serum prostate-specific antigen (PSA) from 135 patients was detected with this method and compared with enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA).

Results: Each IgG-Au-DNA could be covered with 138 ± 47 oligonucleotides and 11 ± 3 antibodies. The IgG-M could bind 118 μg of antibody per mg. The sensitivity of nano-nucleic acid barcode dot detection technology might allow detection of 1 fg/mL. There were no significant differences in serum PSA from 135 patients when comparing the three methods (compared with ELISA, $r = 0.950$; and with RIA, $r = 0.967$).

Conclusions: The nucleic acid barcode dot method does not require special equipment or complex procedures, but its detection limit is 2–3 orders of magnitude lower than ELISA.

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Introduction

At present, techniques that use fluorescence labels and enzyme labels lack adequate sensitivity for early detection of cancer antigens and some neuropeptides (1–3). Although the sensitivity of detection using isotopes may reach 1 ng/mL, this requires special equipment and safeguards limiting its application (4).

Polymerase chain reaction (PCR) technology has been used for the amplification and detection of trace amounts of DNA (5). This technology can theoretically detect single stranded DNA and has good specificity (6). However, protein molecules are different from DNA molecules. The number of protein molecules cannot be increased using amplification techniques, which limits sensitivity (1). In 1992, Sano et al. (7) combined an immunoassay with PCR, creating a high sensitive detection technology, immuno-PCR. A specific DNA is attached to antibody using a joining molecule, and a corresponding relationship is established between antigen and DNA. Thus, protein detection equates to nucleic acid detection. However, the oligonucleotide ligation antibody technique is complex, and the number of oligonucleotides attached to antibodies is small, limiting sensitivity. In 2003, Mirkin et al. (8) amplified oligonucleotides on gold nanoparticle bio-probes using PCR. Ultramicro concentration of protein is measured by detecting the amount of oligonucleotides. However, this method is dependent on oligonucleotides being fixed on a glass chip requiring special equipment, which limits its application.

We introduce a method for ultramicro detection of protein based on nucleic acid barcode dot technology. Hydrosulfo, biotinylated oligonucleotide (Bio-ssDNA-SH) and rabbit anti-human prostate-specific antigen (PSA) polyclonal antibody were combined with gold nanoparticles (30 nm) to prepare dual-labeled gold nanoparticle bio-probes (IgG-Au-DNA) (9). Magnetic micro-spheres are connected with PSA monoclonal antibodies to produce magnetic probes (IgG-M). IgG-M reacts with antigens in serum, IgG-Au-DNA is added, and then the antigen-antibody complex undergoes magnetic separation. Bio-ssDNA-SH on micro-spheres was diluted with dithiothreitol (DTT) and coated onto a nitrocellulose membrane. Bio-ssDNA is detected by the reaction of alkaline phosphatase-labeled streptavidin (SAAP). Detection of anti-

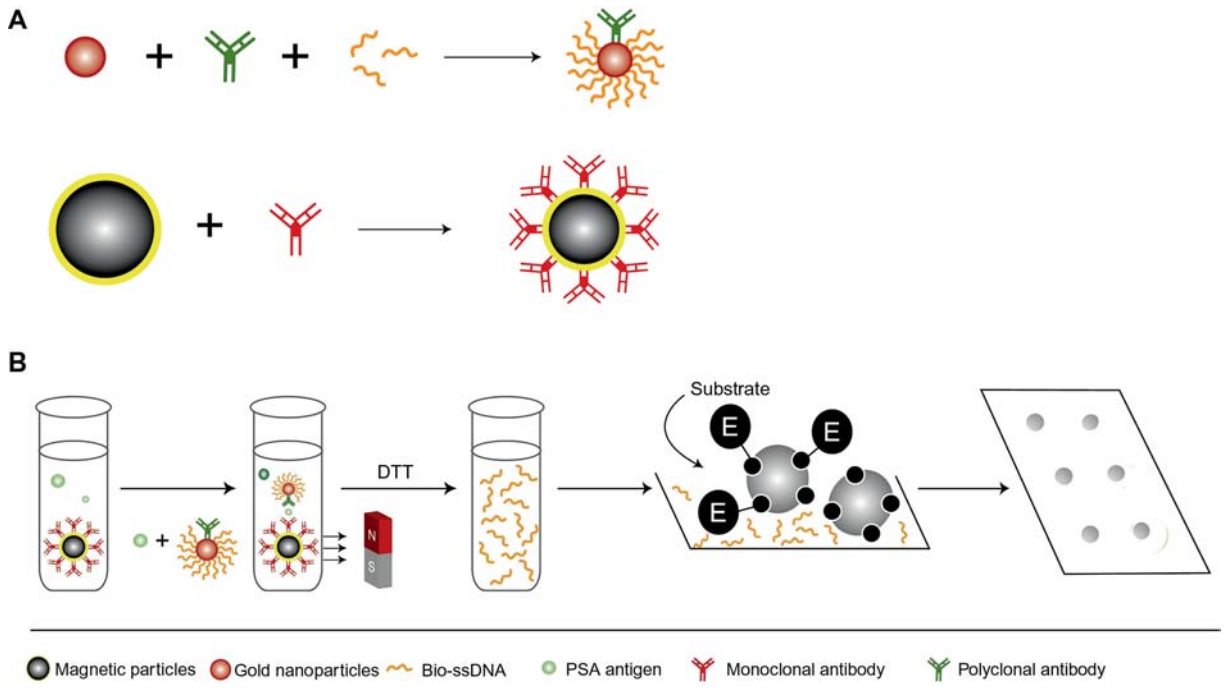

Figure 1 Nucleic acid code amplification assay.
(A) Preparation of the dual-labeled gold nanoparticle bio-probes and magnetic probe. (B) PSA detection scheme (DTT, dithiothreitol; E, enzyme).

gen is performed using nucleic acid barcode detection. Sensitivity to 1 fg/mL may be achieved without use of PCR (Figure 1). This method was used for the measurement of serum PSA and compared with enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) methods.

Materials and methods

Preparation of IgG-Au-DNA (9)

Five mL of nano-gold colloid (3.5 nmol/L) was adjusted to pH 9.0 with Na_2CO_3. Goat antihuman PSA IgG (35 μg) (Lab Vision Corporation, Fremont, USA) and 1000 μL of 3′-terminal thiol-modified oligonucleotide (13.2 μg/mL, Shanghai Sangon Biological Engineering Technology Services Co. Ltd, Shanghai, China) were added and mixed for 3 h at 4°C. Next, 500 μL of phosphate buffered saline (PBS) (pH 7.4, 0.01 mol/L) and 5 μL of 10% SDS were added and mixed for 30 min. One mL of NaCl (1.2 mol/L) was gradually added and allowed to stand for 3 h. Finally, 0.5 mL of 5% bovine serum albumin (BSA) was added and stored in the dark at 4°C.

Preparation of IgG-M

One hundred mg of sodium polymannuronate was dissolved in 4 mL of purified water and then 2 mL of 5% Fe_3O_4 magnetic fluid (mean diameter: 200 nm, The Research Institute of Micro/Nano Science and Technology, Shanghai Jiao Tong University, Shanghai, China) was added and mixed at 42°C followed by ultrasound for 15 min. The mixture was adjusted to pH 10.0 with 10% Na_2CO_3, and heated to 60°C. Using ultrasound and high speed stirring, the mixture was added to 90 mL of AOT/normal heptane oil at 60°C forming a transparent gray-black reverse microemulsion system. Using ultrasound and stirring, 4.5 mL of 30% $CaCl_2$ was added to the microemulsion, followed by magnetic separation, and then washing with acetone, alcohol and distilled water, respectively. Following centrifugation and vacuum freeze-drying, the resultant material was stored.

One mg of prepared magnetic spheres was removed, washed three times with phosphate buffer, and then 0.01 mol/L PBS (pH 7.0) was added to produce a final volume of 4 mL. Five mg of water soluble carbodiimide (EDC) and 7.5 mg of sufl-NHS were added and mixed for 15 min at room temperature. Next, 50 mg of 6-aminocaproic acid was added and stirred for 3 h. We then added 600 μL of PSA monoclonal antibodies (30 μg/mL; Lab Vision Corporation, USA) followed by stirring for 6 h, then sealed with 1 mL of glycine solution (0.2 M) containing 0.2% BSA, and finally magnetic separation before storing at 4°C. The shape was observed with a transmission electron microscope (TEM) and light microscope.

Nucleic acid barcode dot detection method

Forty μL of magnetic spheres (5 mg/mL), and 20 μL of PSA standard solution was prepared by diluting 13 μg of the PSA standard with PBS (pH 7.2) containing 0.2% BSA in a 1 mL volume at 10 μg/mL, 1 μg/mL, 100 ng/mL, 1 ng/mL, 10 pg/mL, 100 fg/mL or 1 fg/mL, or 20 μL of test serum diluted with PBS (pH 7.2) containing 0.2% BSA to 1:50. These were incubated at 37°C for 1 h followed by magnetic separation, then washed three times with 0.01 mol/L PBS (pH 7.4) containing 0.2% BSA, and then 80 μL of gold nanoparticle probes (10 nmol/L) was added at 37°C for 1 h followed by magnetic separation, then washed three times with 0.01 mol/L PBS (pH 7.4) containing 0.2% BSA. To a volume of

50 μL, 5 μL of DTT (Sigma Company, St. Louis, USA) was added to make the final concentration be 0.01 mol/L, DNA was dissociated for 3 h followed by magnetic separation and ultracentrifugation at 8944 *g* for 5 min at low temperature. Next, 10 μL of separated DNA barcode was coated on a 5×8 cm nylon membrane and baked at 60°C for 30 min. SAAP (dilution 1:100; Lab Vision Corporation, USA) was then added and 30 min after reaction at room temperature, washed with 0.01 mol/L PBS (pH 7.4) three times. This was followed by addition of NBT/BCIP (nitroblue tetrazolium/5-bromochloro-3 indole phosphate-4) in the dark for development for 20 min. Superfluous water was absorbed and then allowed to dry. Visual assessment was used to compare dots. The dots can be analyzed with quantitative analysis software to calculate the amount of PSA by comparing the dot values from different standard samples. We calculated the mean value of three repeated square matrixes.

Clinical serum specimens and PSA detection

Serum was obtained from 45 patients aged 49–72 years (mean 61 years) diagnosed with prostate cancer by pathological examination; 45 patients with prostatic hyperplasia aged 51–70 years (mean 58 years); 15 patients, eight men and seven women, with lung cancer (n=6), rectal cancer (n=5) or breast cancer (n=4), aged 40–67 years (mean 53 years); and from 30 patients undergoing heath examination (18 men and 12 women) aged 40–65 years (mean age 50 years). All study methods were consistent with the Helsinki Declaration of 1975 as revised in 1996. Serum specimens were stored at −80°C for future use. Serum was diluted with PBS (1% BSA) to 1:50, and then 20 μL was used. Testing was performed in triplicate. The background values obtained by nucleic acid barcode detection were compared with a standard band. PSA concentrations >4 ng/mL were considered positive and PSA concentrations ≤4 ng/mL were considered negative to determine the specificity and sensitivity of nucleic acid barcode detection for serum PSA. The results obtained by nucleic acid barcode detection also were compared with those obtained with by ELISA (CanAg Diagnostic AB, Gothenburg, Sweden) and RIA kit (CIS BIO Inc, France). Paired χ^2-test was used in the test result analysis.

Statistical analysis

SPSS13 software was used for the statistical analysis of all data. Qualitative results were expressed as $\bar{\chi} \pm s$. The χ^2-test was used to compare quantitative data. Statistical significance was established as $p<0.05$. Since data were not normally distributed, Spearman correlation analysis was used for the results obtained by the nucleic acid barcode dot method, ELISA and RIA. The α value was set at 0.05.

Results and discussion

Characteristics of IgG-Au-DNA

This method requires two types of probes. The IgG-Au-DNA probe has a diameter of 30±5 nm with uniform size assessed by TEM and laser light scattering (9). Gold nanoparticles may be stored in various types of buffer solutions because of changes in its stability which helps avoid false positive tests. The amount of IgG covering the gold nanoparticles can be detected using Coomassie brilliant blue (G-250) and calculated. The number of IgG molecules covering each IgG-Au-DNA was 11±3. Thiol-modified oligonucleotides combined with gold nanoparticles were used as target molecules. The number of nucleic acid barcode on each probe is important because detected nucleic acid barcodes were in direct proportion to measured signal. The number of fluorescin isothiocynate (FITC)-labeled and thiol-modified oligonucleotides on gold nanoparticles replaced by DTT were calculated with a fluorospectrophotometer. The maximum dose of oligonucleotide on 1 mL of gold nanoparticles is about 24.3 μL/mL (165 μg/mL). The number of oligonucleotide on each gold nanoparticle was 138±47.

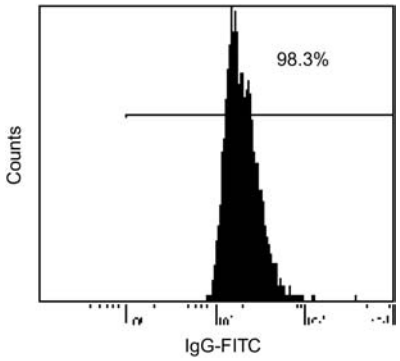

Figure 2 Immunoreactive antibodies in magnetic probes analyzed by flow cytometry.

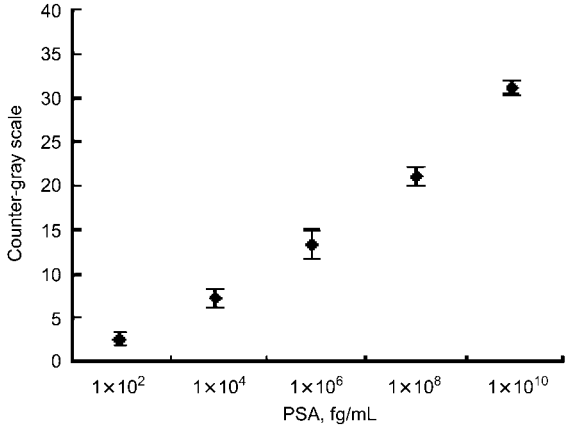

Figure 3 Standard curve for PSA analysis.

Characteristics of IgG-M

The cross-linked antibody on the magnetic microsphere combines with antigen, followed by magnetic separation. However, the affinity interaction (such as antigen-antibody reaction) between biomolecules possesses steric specificity, and steric hindrance usually makes the separated biomacromolecules not able to bind with ligands resulting in poor separations efficiency. Therefore, 6-aminocaproic acid is used as an ''arm'' molecule to connect with the surface of microspheres. The ''arm'' is connected with antibodies by cross-linked EDC to prepare excellent magnetic microsphere

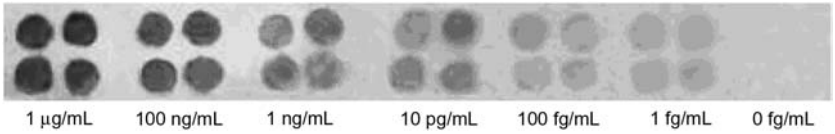

Figure 4 Diagram of PSA test standards on Nylon membrane (4 dots in each concentration).

probes. One milligram of magnetic micro-spheres may connect with up to 118 μg of antibodies. Flow cytometry indicated that there were immunoreactive antibodies in 98.3% of the IgG-M (Figure 2). Big magnetic micro-spheres do not necessarily have good separation, and may yield false positive results. IgG-M with 200 nm-mean diameters can achieve good separation.

Nano-nucleic acid barcode dot detection

PSA was studied due to its important role in prostate cancer and breast cancer. In patients with prostate cancer, PSA level was very low following surgery. Also, PSA may be used as an indicator of tumor recurrence and response to chemotherapy (10). In addition, PSA is an important target in screening for breast cancer. Under normal conditions, serum PSA is much lower in women than in men. Ultra-sensitive test methods for PSA are important in screening and diagnosis of breast cancer (11). For detection of PSA, the molecule was captured using a sandwich method with IgG and IgG-Au-DNA. The compound underwent magnetic separation in solution. The DNA barcode was released with DTT and detected directly without the requirement for PCR. BiossDNA was detected with SAAP. The results were obtained by visual examination. At the same time, a standard curve from 1 fg to 1 μg/mL (Figures 3 and 4) was produced, and PSA concentrations from 100 fg/mL to 1 μg/mL showed good linear relationship ($R=0.9759$). The sensitivity for analysis obtained from the standard curve was 1 fg/mL. The semi-quantitative value of the specimen was determined by visual comparison of specimens with the standard curve. The color scale of the specimens may also be analyzed with software to obtain the amount of PSA when one knows the DNA barcode in the antigen-antibody complex.

In this method, antigen was detected with two types of probes using nucleic acid fragment detection. On each IgG-Au-DNA, protein combines with many oligonucleotides, and then combines with biotin-streptavidin which greatly increases the sensitivity of the method. The lower limit of PSA in this method was 100 fg/mL, which is 1000 times more sensitive than ELISA. Compared with immunodetection (1 ng/mL), the method does not require specialized equipment and safeguards (12). Also, the method is simple and quick. Sensitivity is increased because of more effective capture steps and signal background is reduced because the DNA barcode is detected directly.

Serum PSA detection

Serum specimens from 135 cases (prostate cancer group, prostatic hyperplasia group, other tumor group and normal control group) were detected with our method, ELISA and RIA. PSA >4 ng/mL was considered as positive and PSA ≤ 4 ng/mL was considered negative. χ^2-test indicated $p>0.05$. There were no significant differences in PSA between the three methods at $\alpha=0.05$.

Since data were not normally distributed, Spearman correlation analysis was used for the PSA results obtained by nucleic acid barcode dot method, ELISA and RIA (Figures 5 and 6). Results indicated that the coefficient correlations (r) of nano-nucleic acid barcode dot detection method with ELISA and RIA were 0.950 ($p>0.001$) and 0.967 ($p>0.001$), respectively.

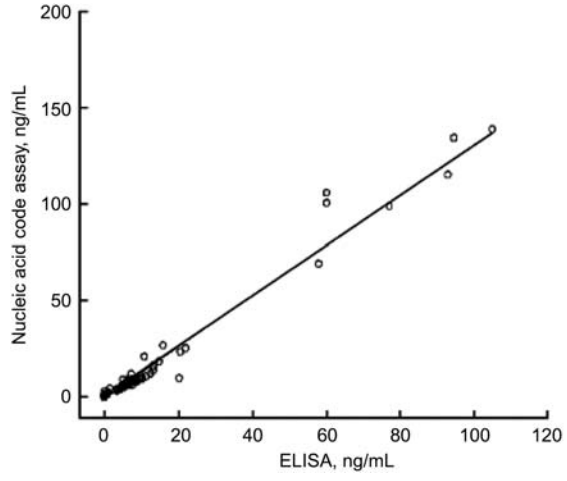

Figure 5 Correlation between nucleic acid barcode assay and ELISA.

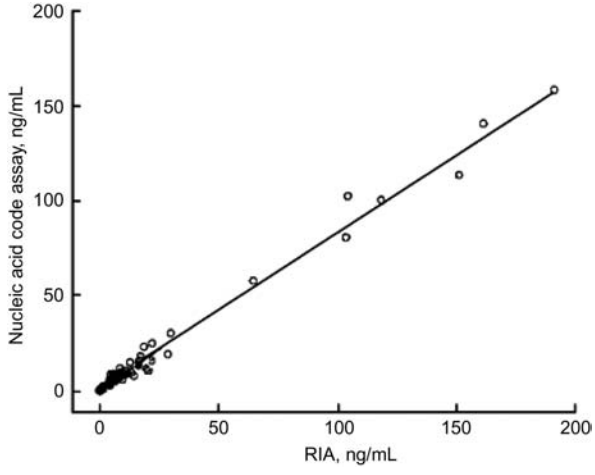

Figure 6 Correlation between nucleic acid barcode assay and RIA.

Conclusions

An ultramicro detection method for protein was established using nucleic acid barcode as a marker. The number of DNA barcodes on bioprobes may be adjusted by the size and surface of the gold nanoparticles. The detection limit of this method is 2–3 orders of magnitude lower than ELISA. This method does not need special equipment or complex procedures. It only requires probes, magnetic separators and nylon membrane. Visual assessment or image analysis software can be used to obtain results with this method. We provide a technology for detection of ultramicro amounts of protein, with great potential for different applications.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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